Supplementary Material (ESI) for Chemical Communications

Towards Preparative-Scale, Biocatalytic Alkene Reductions

Supporting Information

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General

Reagents were obtained from commercial suppliers and used as received. LB medium contained Bacto-Tryptone (1%), Bacto-Yeast Extract (0.5%) and NaCl (1%). Agar (1.5%) was used to form solid medium when required. GC/MS analyses were performed on a Chirasil-Dex CB column (25 m × 0.25 mm) using a mass-selective detector (EI, 70 eV). The temperature program involved 60°C (2 min) followed by a 10°C / min increase to 180°C (10 min). Under these conditions, peaks eluted at 10.5 min (citronellal), 11.8 min (neral), 12.2 min (geranial) and 12.5 min (cintronellol). Chiral analyses were performed on a BetaDex column (30 m × 0.25 mm). The temperature program involved 95°C (35 min) followed by a 5°C / min increase to 160°C (2 min), then a 10°C / min increase to 200°C (5 min). Under these conditions, peaks eluted at 26.6 min ((*S*)-citronellal), 27.0 min ((*R*)-citronellal), 36.6 min ((*S*)-citronellol), 36.9 min ((*R*)-citronellol), 43.4 min (neral) and 46.2 min (geranial). Optical rotations were measured with a Perkin-Elmer 241 polarimeter operating a room temp. GST-OYE 2.6 was purified with High-Affinity GST resin (GenScript) as described earlier.[1] Total protein concentrations were determined by Bradford assays using BSA as a standard.[2] OYE catalytic activity was determined against 2-cyclohexenone.[1]

Geranial 1. Geraniol (33.4 mmoles, 5.25 g) was stirred with MnO_2 (125 mmoles, 10.85 g) in 30 mL of CH_2Cl_2 at room temp. After 24 hr, additional MnO_2 was added (63 mmoles, 5.47 g) and stirring was continued overnight. Analysis by GC/MS showed that all starting material had been consumed. Solids were removed by filtration through a silica pad, then solvent was removed under vacuum to yield the title compound (4.72 g, 89% yield) as a bright yellow oil, which was stored under Ar at -20°C. GC analysis of the final product showed 96% geranial and 4% neral.

Neral 4. Nerol (136 mmoles, 21.0 g) was stirred with MnO_2 (500 mmoles, 43.40 g) in 150 mL of CH_2Cl_2 at room temp. After 22 hr, additional MnO_2 was added (125 mmoles, 10.85 g) and stirring was continued for an additional 4 hr. Analysis by GC/MS showed that all starting material had been consumed. Solids were removed by filtration through a silica pad, then solvent was removed under vacuum to yield the title compound (20.30 g, 98% yield) as a yellow oil, which was stored under Ar at -20°C. GC analysis of the final product showed 97% neral and 3% geranial.

Preparation of *P. stipitis* GST-OYE 2.6 crude extract. *E. coli* BL21(DE3) cells overexpressing OYE 2.6 were grown on LB plates supplemented with 200 μg/mL ampicillin. A single colony was used to inoculate 40 mL of LB medium containing 200 μg/mL ampicillin. After overnight growth at 37°C, this was added to 4 L of LB medium supplemented with 80 mL of sterile 20% glucose, 2 g/L ampicillin and 0.5 mL of Sigma AF 204 antifoam. Cells were grown in a New Brunswick M19 fermenter at 37°C with stirring and airflow maintained at 700 rpm and 4 L/min, respectively. After 2 hr, the $O.D._{600}$ value reached 0.6. The culture was cooled to 30°C and protein overexpression was induced by adding 0.48 mL of sterile 840 mM IPTG solution. The culture conditions were maintained for an additional 4 hr, when the $O.D._{600}$ value reached 4.2. Cells were harvested by centrifugation (6,500 × *g* at 4°C) to yield 28.2 g (wet cell weight) of biomass that could be stored at -20°C or used directly.

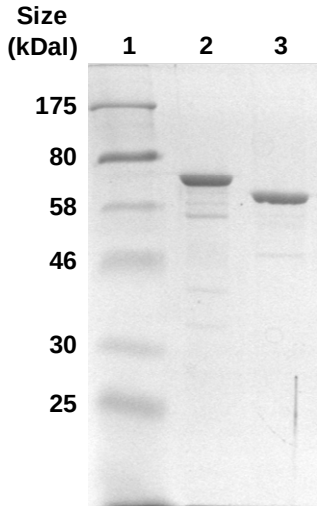
Cells (*ca*. 30 g) were resuspended in 30 mL of 100 mM KP_i, pH 7.5, then lysed by two passages through a chilled French pressure cell in the presence of 10 μM PMSF. Debris was removed by centrifugation (20,000 × *g* for 45 min at 4°C), then the supernatant (44.5 mL) was slowly mixed with 15 mL of saturated $(NH_4)_2SO_4$ to yield a final value of 25% saturation. After stirring gently for 10 min at 4°C, insoluble material was removed by centrifugation (20,000 × *g*

for 45 min at 4°C). Solid $(NH_4)_2SO_4$ (5.0 g) was slowly dissolved in the supernatant (55 mL) by stirring at 4°C. After an additional 10 min, insoluble proteins were removed by centrifugation (20,000 × *g* for 45 min at 4°C) and the supernatant was discarded. The pellet was resuspended in 25 mL of 100 mM KP_i, pH 7.5, then insoluble material was removed by centrifugation as described above to yield 29 mL of protein solution that was frozen in aliquots at -20°C until needed. Activity assays showed 7.9 U/mL (2-cyclohexenone substrate).

Preparation of *E. coli* GST-NemA crude extract. *E. coli* BL21(DE3) cells overexpressing NemA were grown on LB plates supplemented with 200 μg/mL ampicillin. Using the procedure described above, cells were grown, induced at $O.D._{600}$ = 0.8 and harvested to yield 28.15 g of cells (wet weight). The biomass was suspended in 30 mL of 100 mM KP_i, pH 7.5, lysed by two passages through a chilled French pressure cell and clarified by centrifugation (20,000 × *g* for 45 min at 4°C) to afford 45 mL of crude lysate that showed an activity of 44 U/mL (2-cyclohexenone substrate).

SDS-PAGE analysis of purified *S. stipitis* OYE 2.6 and *E. coli* NemA fusion proteins. Purified proteins were separated on a 10% polyacrylamide gel, then proteins were stained with Coomassie blue. Theoretical values: GST-OYE 2.6, 71.3 kDal; GST-NemA, 65.5 kDal.

Lane	Sample
1	M.W. Standards
2	GST-OYE 2.6
3	GST-NemA



CLEA preparation. Purified GST-OYE 2.6 (17 mg, 0.50 mL) was mixed with purified GDH-102 (6 mg) and 0.50 mL of 100 mM KP_i, pH 7.5. A 0.50 mL aliquot was transferred to a microcentrifuge tube and 0.50 mL of saturated $(NH_4)_2SO_4$ solution was added. The tube was rotated gently at 4°C for 15 min, then 384 mg of solid $(NH_4)_2SO_4$ was added the tube was rotated gently at 4°C for an additional 15 min. Glutaraldehyde (15 μL, 50% aqueous solution) was added to a final concentration of 75 mM and the tube was rotated gently at 4°C for an additional 2 hr. The CLEA was collected by centrifugation and washed three times with cold 100 mM KP_i, pH 7.5.

Acetylation of GST-OYE 2.6. Four microcentrifuge tubes containing 4 mg of purified GST-OYE 2.6 in a volume of 0.50 mL were prepared (1 – 4). Sodium acetate (190 mg, 50% aqueous solution) was added to tubes 2 and 4. Neat acetic anhydride (1 μL) was added to tubes 3 and 4. All tubes were gently rotated at 4°C for 1 hr, then samples 2 – 4 were dialyzed against 100 mM KP_i, pH 7.5, 50 mM NaCl, 50% glycerol for 3 hr. Aliquots (250 μL) from samples 1 – 4 were transferred to microcentrifuge tubes and citronellal was added to a final concentration of 25 mM (28 μL of a 250 mM stock in EtOH) and gently rotated overnight at 4°C. Each sample, along with a control that was not treated with citronellal was diluted 1 : 10 with 100 mM KPi, pH 7.5 and assayed for protein concentration and catalytic activity. The latter quantities were measured by incubating 2.5 mM 2-cyclohexenone (from a X M EtOH stock solution) with 100 mM KP_i, pH 7.5 that contained 0.2 mM NADPH and an appropriate quantity of protein in a total volume of 1.0 mL. The change in A_{340} was measured at 25°C. The small change in A_{340} observed during the same time period in a control reaction lacking 2-cyclohexenone was subtracted from all of the determinations.

	Specific Activity (U / mg)			
	Untreated	NaOAc	Ac_2O	NaOAc, Ac_2O
Control	1.75 ± 0.17	1.43 ± 0.08	0.12 ± 0.01	1.02 ± 0.04
25 mM citronellal	1.47 ± 0.09	1.46 ± 0.13	0.26 ± 0.02	0.84 ± 0.04

References

1. D. J. Bougioukou, Ph.D. thesis, University of Florida, 2006.

2. M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248-254.